Fig. 1

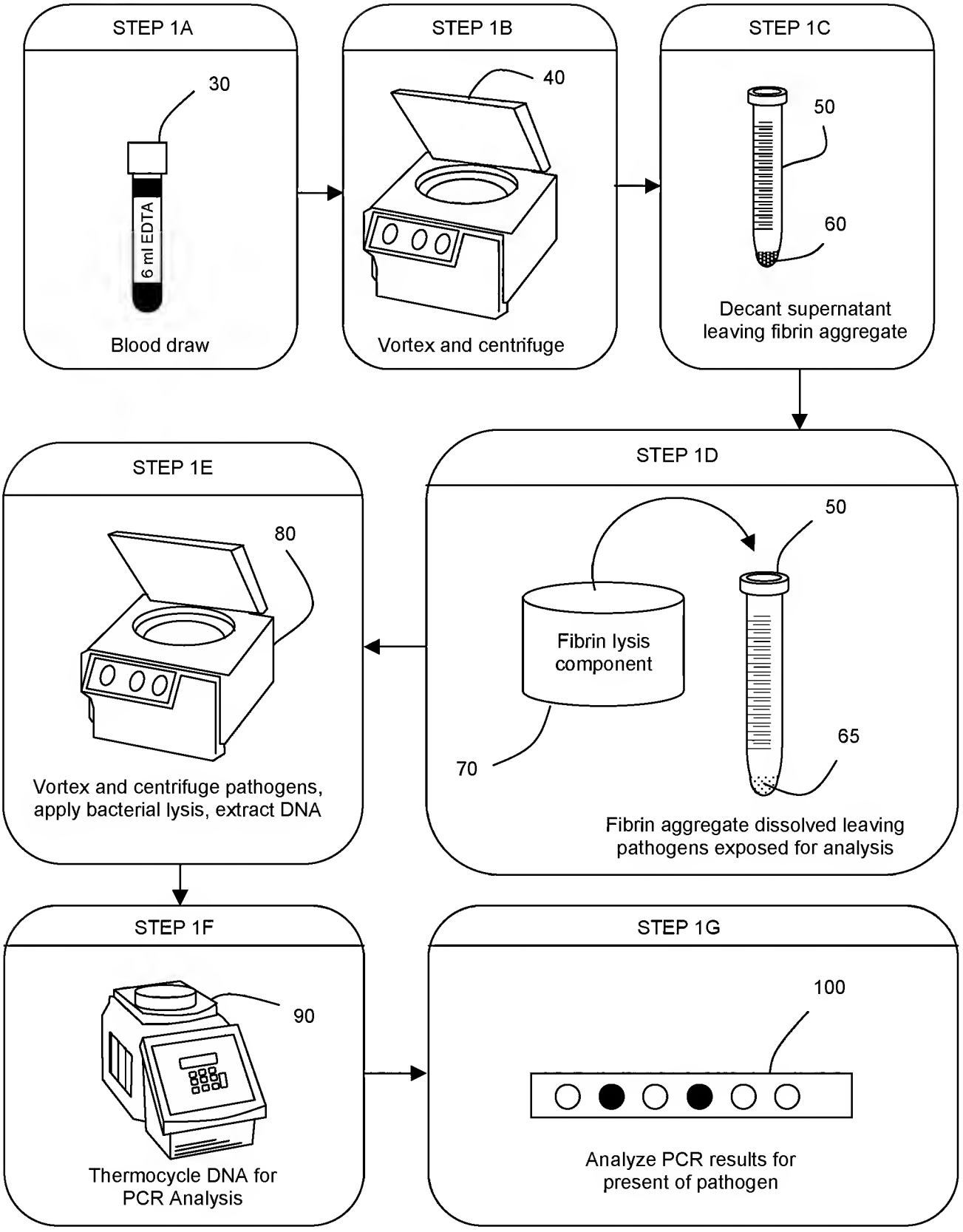
STEP 1A
30
6 ml EDTA
Blood draw
STEP 1B
40
Vortex and centrifuge
STEP 1C
50
60
Decant supernatant leaving fibrin aggregate
STEP 1D
50
Fibrin lysis component
70
65
Fibrin aggregate dissolved leaving pathogens exposed for analysis
STEP 1E
80
Vortex and centrifuge pathogens, apply bacterial lysis, extract DNA
STEP 1F
90
Thermocycle DNA for PCR Analysis
STEP 1G
100
Analyze PCR results for present of pathogen

Fig. 2

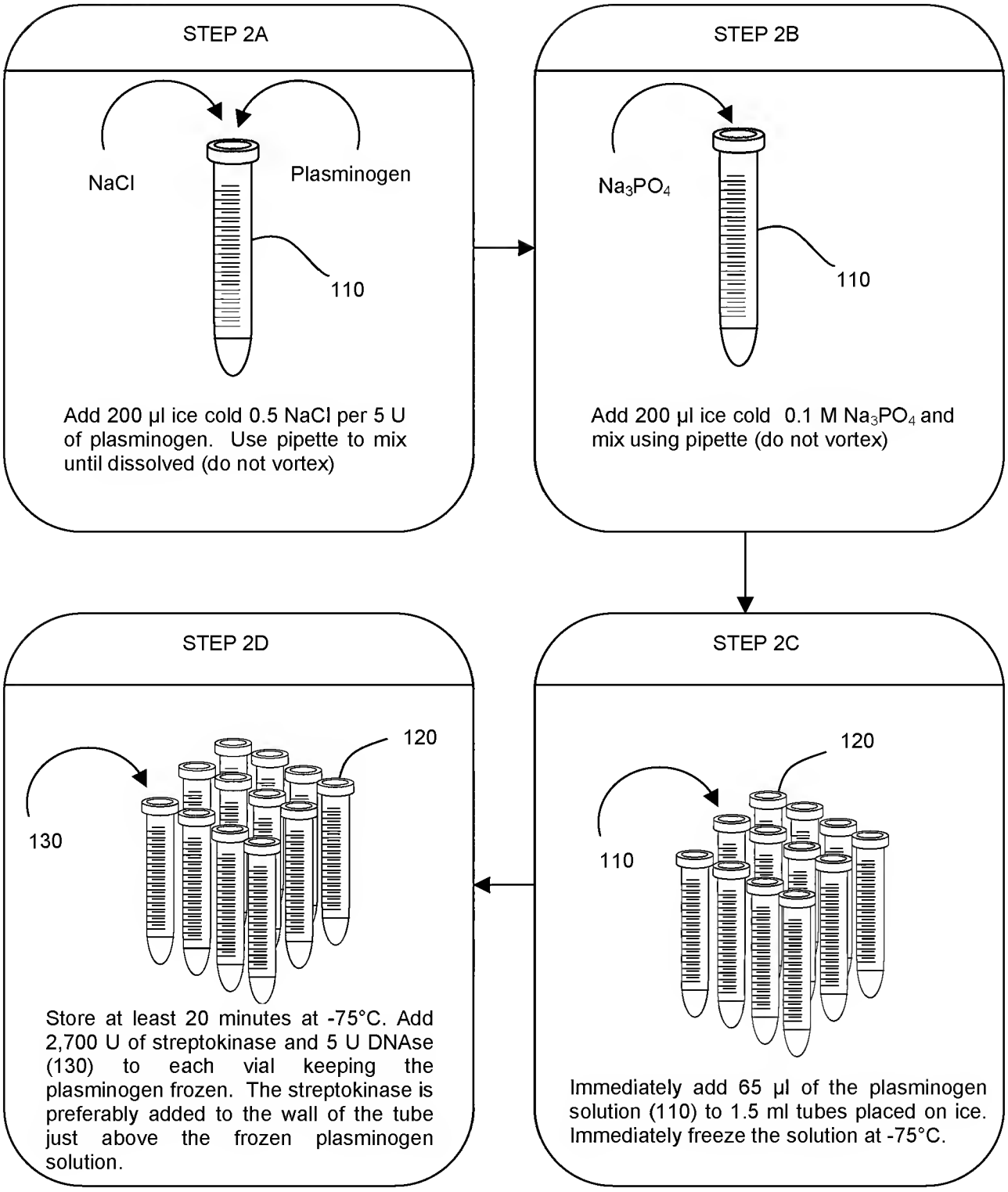
STEP 2A
NaCl
Plasminogen
110
Add 200 µl ice cold 0.5 NaCl per 5 U of plasminogen. Use pipette to mix until dissolved (do not vortex)
STEP 2B
Na_3PO_4
110
Add 200 µl ice cold 0.1 M Na_3PO_4 and mix using pipette (do not vortex)
STEP 2C
120
110
Immediately add 65 µl of the plasminogen solution (110) to 1.5 ml tubes placed on ice. Immediately freeze the solution at -75°C.
STEP 2D
120
130
Store at least 20 minutes at -75°C. Add 2,700 U of streptokinase and 5 U DNAse (130) to each vial keeping the plasminogen frozen. The streptokinase is preferably added to the wall of the tube just above the frozen plasminogen solution.

Fig. 3

***Bacillus anthracis* Blood Protocol Data Set**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Tested 2 Days Post Spiking
M3200253BA1	36.75	37.76	13.75	Spiked Positive
M3200253BA2	36.59	37.86	13.75	Spiked Positive
M3200253BA3	35.97	38.10	13.75	Spiked Positive
M3200253BA4	37.26	39.53	13.75	Spiked Positive
M3200253BA5	35.36	40.11	13.75	Spiked Positive
M3200253BA6	36.35	45.19	13.75	Spiked Positive
M3200253BA7	36.62	38.64	13.75	Spiked Positive
M3200253BA8	37.04	39.51	13.75	Spiked Positive
M320020BA9	0.00	0.00	0.00	Blank
M/3200226BA1	37.16	39.35	1.38	Spiked Positive
M/3200226BA2	36.79	40.28	1.38	Spiked Positive
M/3200226BA3	37.92	39.94	1.38	Spiked Positive
M/3200226BA4	37.49	40.16	1.38	Spiked Positive
M/3200226BA5	39.66	40.26	1.38	Spiked Positive
M/3200226BA6	39.31	41.19	1.38	Spiked Positive
M/3200226BA7	38.48	40.73	1.38	Spiked Positive
M/320020BA8	0.00	0.00	0.00	Blank

Fig. 4

***Bacillus anthracis* Blood Protocol Data Set: Comparison of Blood from Two Different Individuals and Evaluation of Blood Sample Age**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Extracted 84 Days Post Spiking
V210253BA1	37.73	39.81	10.5	Blood Donor #1
V210253BA2	36.74	39.05	10.5	Blood Donor #1
V210253BA3	36.51	37.99	10.5	Blood Donor #1
V210253BA4	38.12	39.79	10.5	Blood Donor #1
V21020BA5	0.00	0.00	0.00	Blank
M210253BA1	37.86	39.81	2.25	Blood Donor #2
M210253BA2	37.84	39.22	2.25	Blood Donor #2
M210253BA3	37.24	38.52	2.25	Blood Donor #2
M210253BA4	38.68	39.33	2.25	Blood Donor #2
M21020BA5	0.00	0.00	0.00	Blank

Fig. 5

***Bacillus anthracis* Blood Protocol Data Set: Evaluation of Blood Protocol by a Department of Health Laboratorian**

Sample Number	pXO2 Primer / Probes - Crossing Point on Light Cycler	Genomic Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type: All Blood Samples Same Batch as in Table 1
M3200256BA1L	38.81	39.93	13.75	Spiked Positive
M3200256BA2L	36.10	39.26	13.75	Spiked Positive
M/3200223BA3L	36.77	38.58	1.38	Spiked Positive
M320020BA4L	0.00	0.00	0.00	Blank

Fig. 6

***Yersinia pestis* Blood Protocol Data Set**

Sample Number	YP 2 Primer / Probes - Crossing Point on Light Cycler	YP 9 Primer / Probes - Crossing Point on Light Cycler	YP12 Primer / Probes - Crossing Point on Light Cycler	YP 16 Primer / Probes - Crossing Point on Light Cycler	Ave. Calculated CFU/ 6 ml of blood	Comments on Sample Type All Samples Extracted 2 Days Post Spiking
M3180251EYP1	0.00	0.00	0.00	37.97	12.0	Spiked Positive
M3180251EYP2	0.00	47.01	0.00	0.00	12.0	Spiked Positive
M3180251EYP3	41.56	0.00	0.00	40.29	12.0	Spiked Positive
M3180225EYP4	0.00	0.00	0.00	38.98	24.0	Spiked Positive
M3180225EYP6	40.20	44.01	39.66	37.60	24.0	Spiked Positive
M3180251FYP7	0.00	46.15	0.00	39.79	48.0	Spiked Positive
M3180251FYP8	40.48	43.59	41.70	35.47	48.0	Spiked Positive
M3180251FYP9	40.20	41.88	38.67	34.23	48.0	Spiked Positive
M318020YP10	0.00	0.00	0.00	0.00	0.00	Blank

Fig. 7

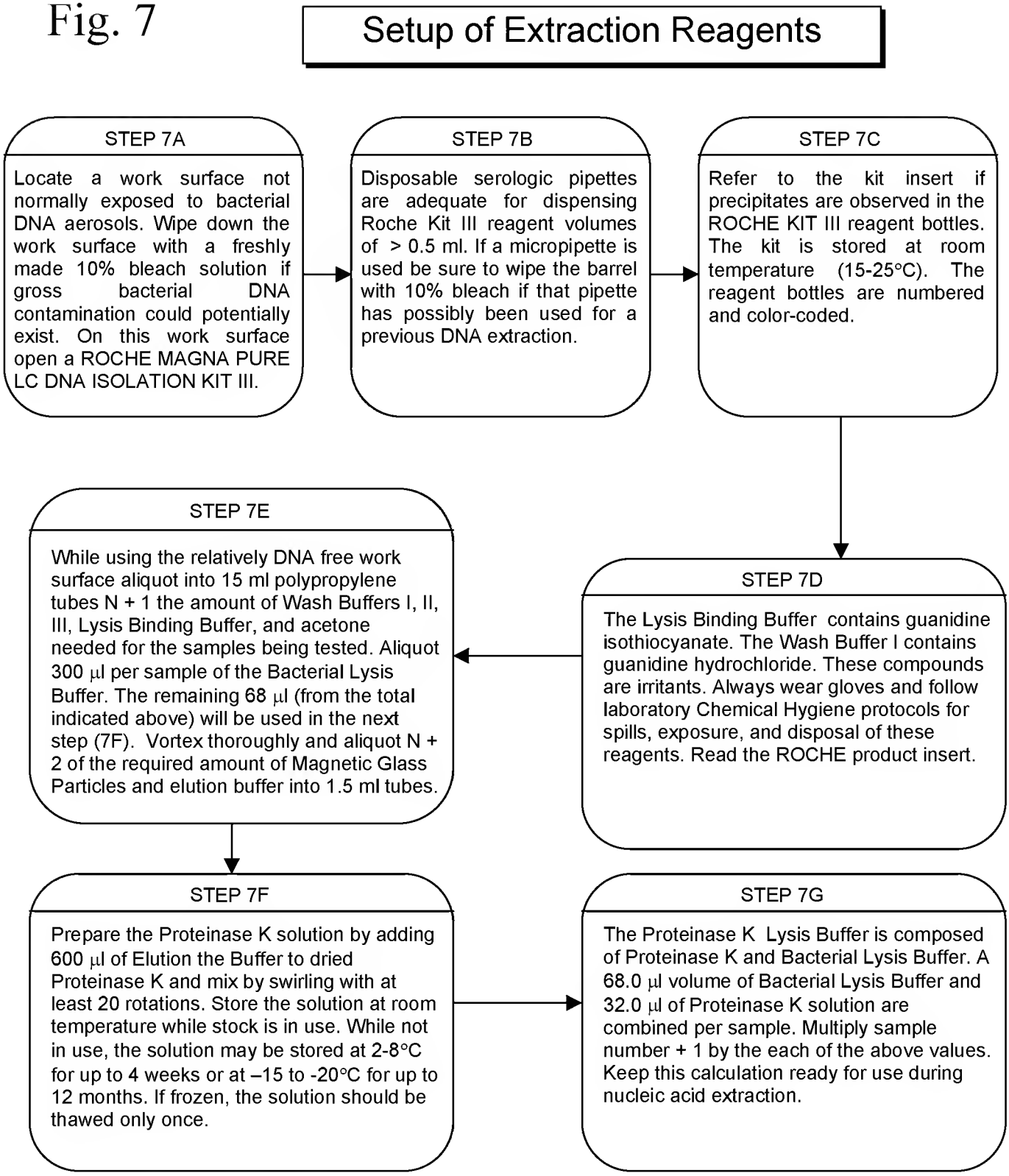
Setup of Extraction Reagents
STEP 7A
Locate a work surface not normally exposed to bacterial DNA aerosols. Wipe down the work surface with a freshly made 10% bleach solution if gross bacterial DNA contamination could potentially exist. On this work surface open a ROCHE MAGNA PURE LC DNA ISOLATION KIT III.
STEP 7B
Disposable serologic pipettes are adequate for dispensing Roche Kit III reagent volumes of > 0.5 ml. If a micropipette is used be sure to wipe the barrel with 10% bleach if that pipette has possibly been used for a previous DNA extraction.
STEP 7C
Refer to the kit insert if precipitates are observed in the ROCHE KIT III reagent bottles. The kit is stored at room temperature (15-25°C). The reagent bottles are numbered and color-coded.
STEP 7D
The Lysis Binding Buffer contains guanidine isothiocyanate. The Wash Buffer I contains guanidine hydrochloride. These compounds are irritants. Always wear gloves and follow laboratory Chemical Hygiene protocols for spills, exposure, and disposal of these reagents. Read the ROCHE product insert.
STEP 7E
While using the relatively DNA free work surface aliquot into 15 ml polypropylene tubes N + 1 the amount of Wash Buffers I, II, III, Lysis Binding Buffer, and acetone needed for the samples being tested. Aliquot 300 µl per sample of the Bacterial Lysis Buffer. The remaining 68 µl (from the total indicated above) will be used in the next step (7F). Vortex thoroughly and aliquot N + 2 of the required amount of Magnetic Glass Particles and elution buffer into 1.5 ml tubes.
STEP 7F
Prepare the Proteinase K solution by adding 600 µl of Elution the Buffer to dried Proteinase K and mix by swirling with at least 20 rotations. Store the solution at room temperature while stock is in use. While not in use, the solution may be stored at 2-8°C for up to 4 weeks or at –15 to -20°C for up to 12 months. If frozen, the solution should be thawed only once.
STEP 7G
The Proteinase K Lysis Buffer is composed of Proteinase K and Bacterial Lysis Buffer. A 68.0 µl volume of Bacterial Lysis Buffer and 32.0 µl of Proteinase K solution are combined per sample. Multiply sample number + 1 by the each of the above values. Keep this calculation ready for use during nucleic acid extraction.

Fig. 8

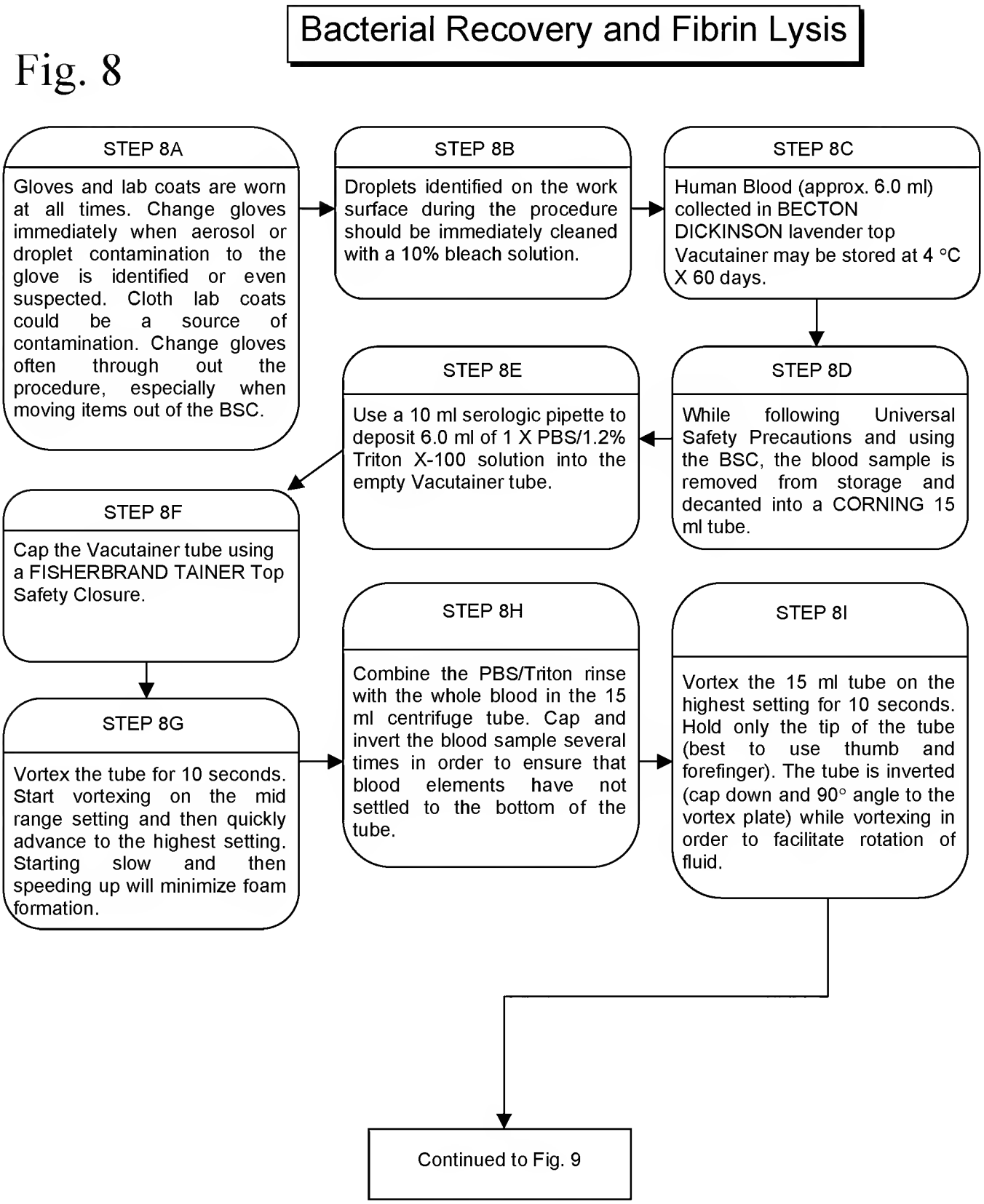
Bacterial Recovery and Fibrin Lysis
STEP 8A
Gloves and lab coats are worn at all times. Change gloves immediately when aerosol or droplet contamination to the glove is identified or even suspected. Cloth lab coats could be a source of contamination. Change gloves often through out the procedure, especially when moving items out of the BSC.
STEP 8B
Droplets identified on the work surface during the procedure should be immediately cleaned with a 10% bleach solution.
STEP 8C
Human Blood (approx. 6.0 ml) collected in BECTON DICKINSON lavender top Vacutainer may be stored at 4 °C X 60 days.
STEP 8D
While following Universal Safety Precautions and using the BSC, the blood sample is removed from storage and decanted into a CORNING 15 ml tube.
STEP 8E
Use a 10 ml serologic pipette to deposit 6.0 ml of 1 X PBS/1.2% Triton X-100 solution into the empty Vacutainer tube.
STEP 8F
Cap the Vacutainer tube using a FISHERBRAND TAINER Top Safety Closure.
STEP 8G
Vortex the tube for 10 seconds. Start vortexing on the mid range setting and then quickly advance to the highest setting. Starting slow and then speeding up will minimize foam formation.
STEP 8H
Combine the PBS/Triton rinse with the whole blood in the 15 ml centrifuge tube. Cap and invert the blood sample several times in order to ensure that blood elements have not settled to the bottom of the tube.
STEP 8I
Vortex the 15 ml tube on the highest setting for 10 seconds. Hold only the tip of the tube (best to use thumb and forefinger). The tube is inverted (cap down and 90° angle to the vortex plate) while vortexing in order to facilitate rotation of fluid.
Continued to Fig. 9

Fig. 9

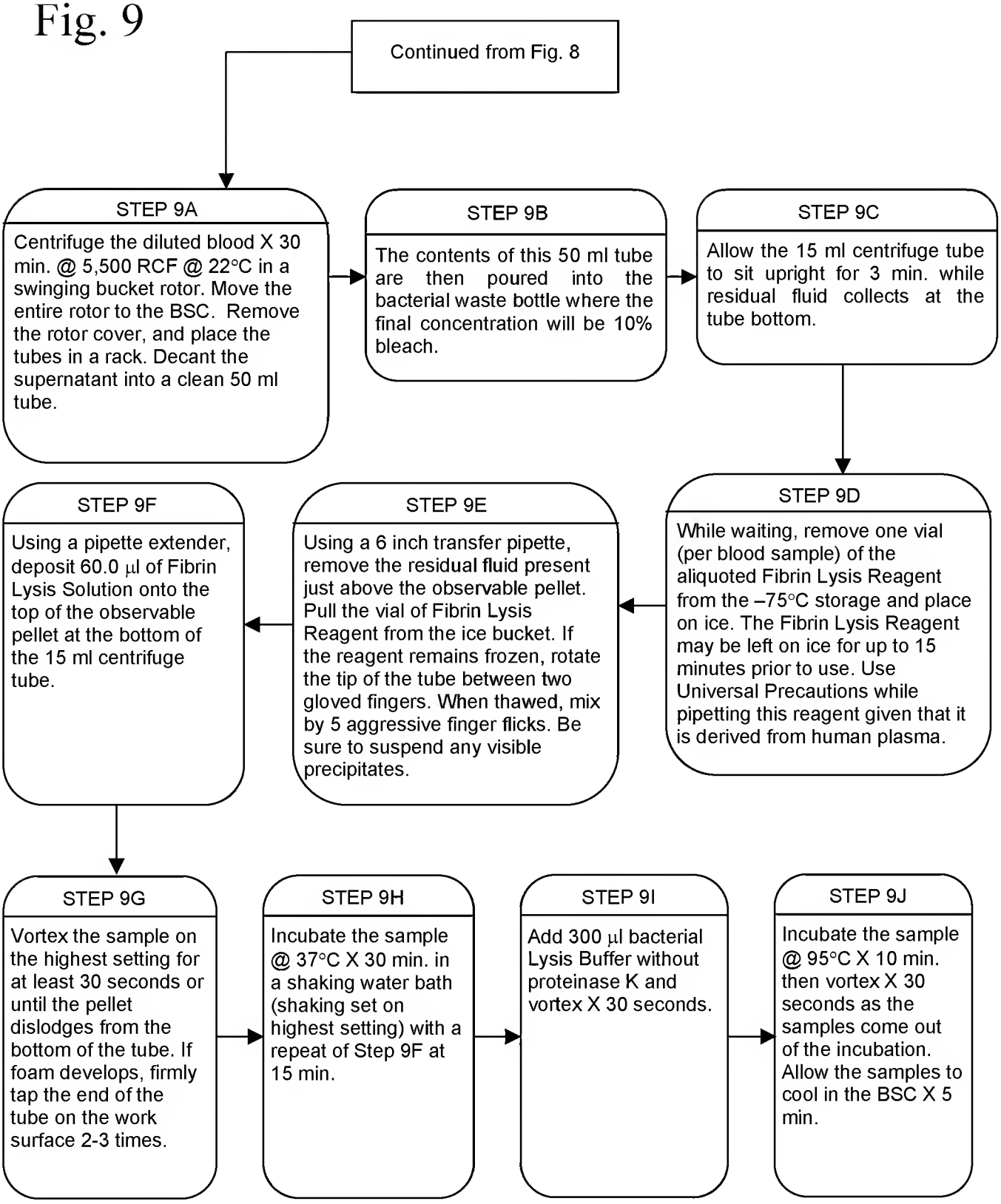
Continued from Fig. 8
STEP 9A
Centrifuge the diluted blood X 30 min. @ 5,500 RCF @ 22°C in a swinging bucket rotor. Move the entire rotor to the BSC. Remove the rotor cover, and place the tubes in a rack. Decant the supernatant into a clean 50 ml tube.
STEP 9B
The contents of this 50 ml tube are then poured into the bacterial waste bottle where the final concentration will be 10% bleach.
STEP 9C
Allow the 15 ml centrifuge tube to sit upright for 3 min. while residual fluid collects at the tube bottom.
STEP 9D
While waiting, remove one vial (per blood sample) of the aliquoted Fibrin Lysis Reagent from the –75°C storage and place on ice. The Fibrin Lysis Reagent may be left on ice for up to 15 minutes prior to use. Use Universal Precautions while pipetting this reagent given that it is derived from human plasma.
STEP 9E
Using a 6 inch transfer pipette, remove the residual fluid present just above the observable pellet. Pull the vial of Fibrin Lysis Reagent from the ice bucket. If the reagent remains frozen, rotate the tip of the tube between two gloved fingers. When thawed, mix by 5 aggressive finger flicks. Be sure to suspend any visible precipitates.
STEP 9F
Using a pipette extender, deposit 60.0 µl of Fibrin Lysis Solution onto the top of the observable pellet at the bottom of the 15 ml centrifuge tube.
STEP 9G
Vortex the sample on the highest setting for at least 30 seconds or until the pellet dislodges from the bottom of the tube. If foam develops, firmly tap the end of the tube on the work surface 2-3 times.
STEP 9H
Incubate the sample @ 37°C X 30 min. in a shaking water bath (shaking set on highest setting) with a repeat of Step 9F at 15 min.
STEP 9I
Add 300 µl bacterial Lysis Buffer without proteinase K and vortex X 30 seconds.
STEP 9J
Incubate the sample @ 95°C X 10 min. then vortex X 30 seconds as the samples come out of the incubation. Allow the samples to cool in the BSC X 5 min.

Fig. 10

Bacterial Lysis and Nucleic Acid Extraction

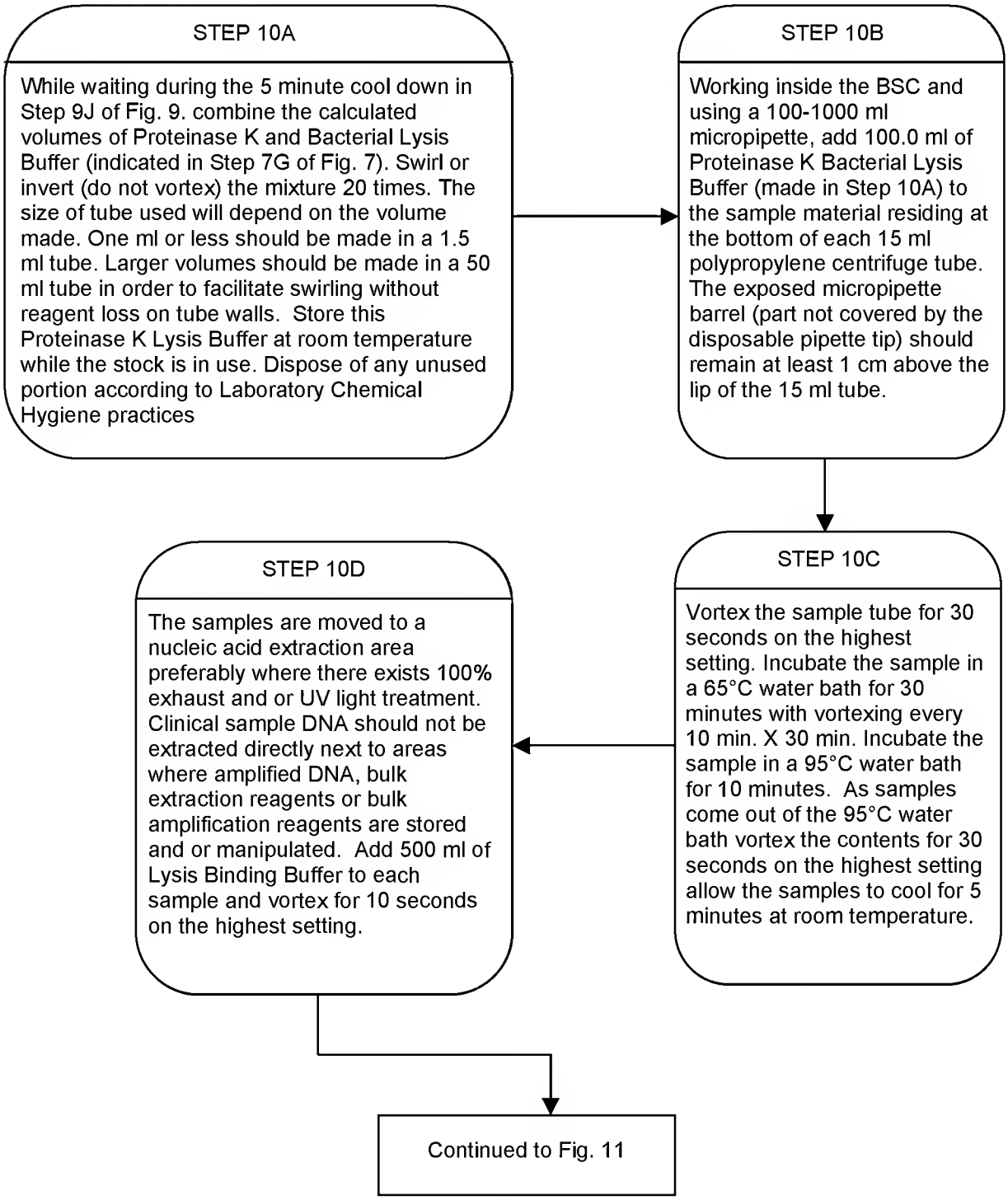

Fig. 11

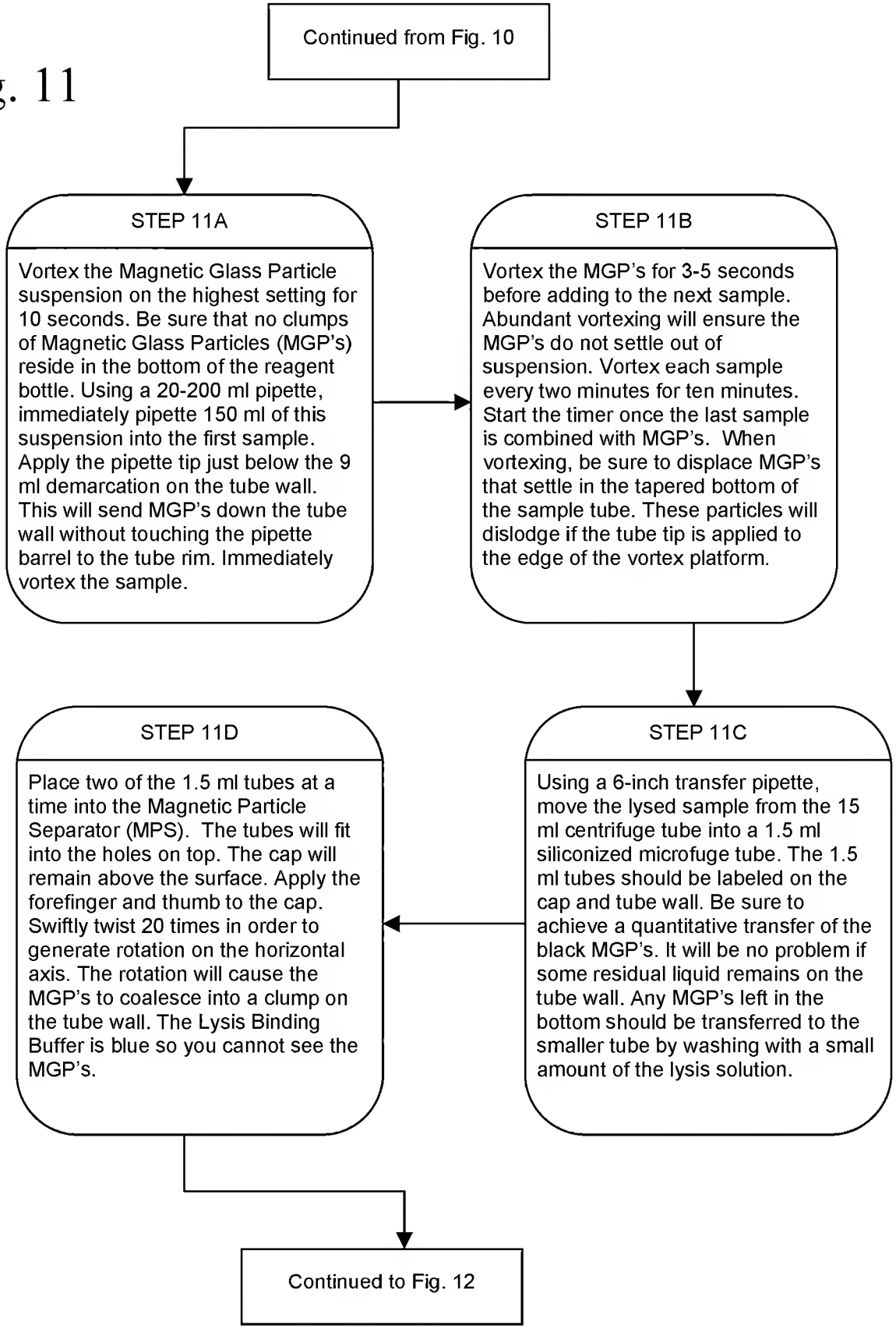
Continued from Fig. 10
STEP 11A
Vortex the Magnetic Glass Particle suspension on the highest setting for 10 seconds. Be sure that no clumps of Magnetic Glass Particles (MGP's) reside in the bottom of the reagent bottle. Using a 20-200 ml pipette, immediately pipette 150 ml of this suspension into the first sample. Apply the pipette tip just below the 9 ml demarcation on the tube wall. This will send MGP's down the tube wall without touching the pipette barrel to the tube rim. Immediately vortex the sample.
STEP 11B
Vortex the MGP's for 3-5 seconds before adding to the next sample. Abundant vortexing will ensure the MGP's do not settle out of suspension. Vortex each sample every two minutes for ten minutes. Start the timer once the last sample is combined with MGP's. When vortexing, be sure to displace MGP's that settle in the tapered bottom of the sample tube. These particles will dislodge if the tube tip is applied to the edge of the vortex platform.
STEP 11C
Using a 6-inch transfer pipette, move the lysed sample from the 15 ml centrifuge tube into a 1.5 ml siliconized microfuge tube. The 1.5 ml tubes should be labeled on the cap and tube wall. Be sure to achieve a quantitative transfer of the black MGP's. It will be no problem if some residual liquid remains on the tube wall. Any MGP's left in the bottom should be transferred to the smaller tube by washing with a small amount of the lysis solution.
STEP 11D
Place two of the 1.5 ml tubes at a time into the Magnetic Particle Separator (MPS). The tubes will fit into the holes on top. The cap will remain above the surface. Apply the forefinger and thumb to the cap. Swiftly twist 20 times in order to generate rotation on the horizontal axis. The rotation will cause the MGP's to coalesce into a clump on the tube wall. The Lysis Binding Buffer is blue so you cannot see the MGP's.
Continued to Fig. 12

Fig. 12

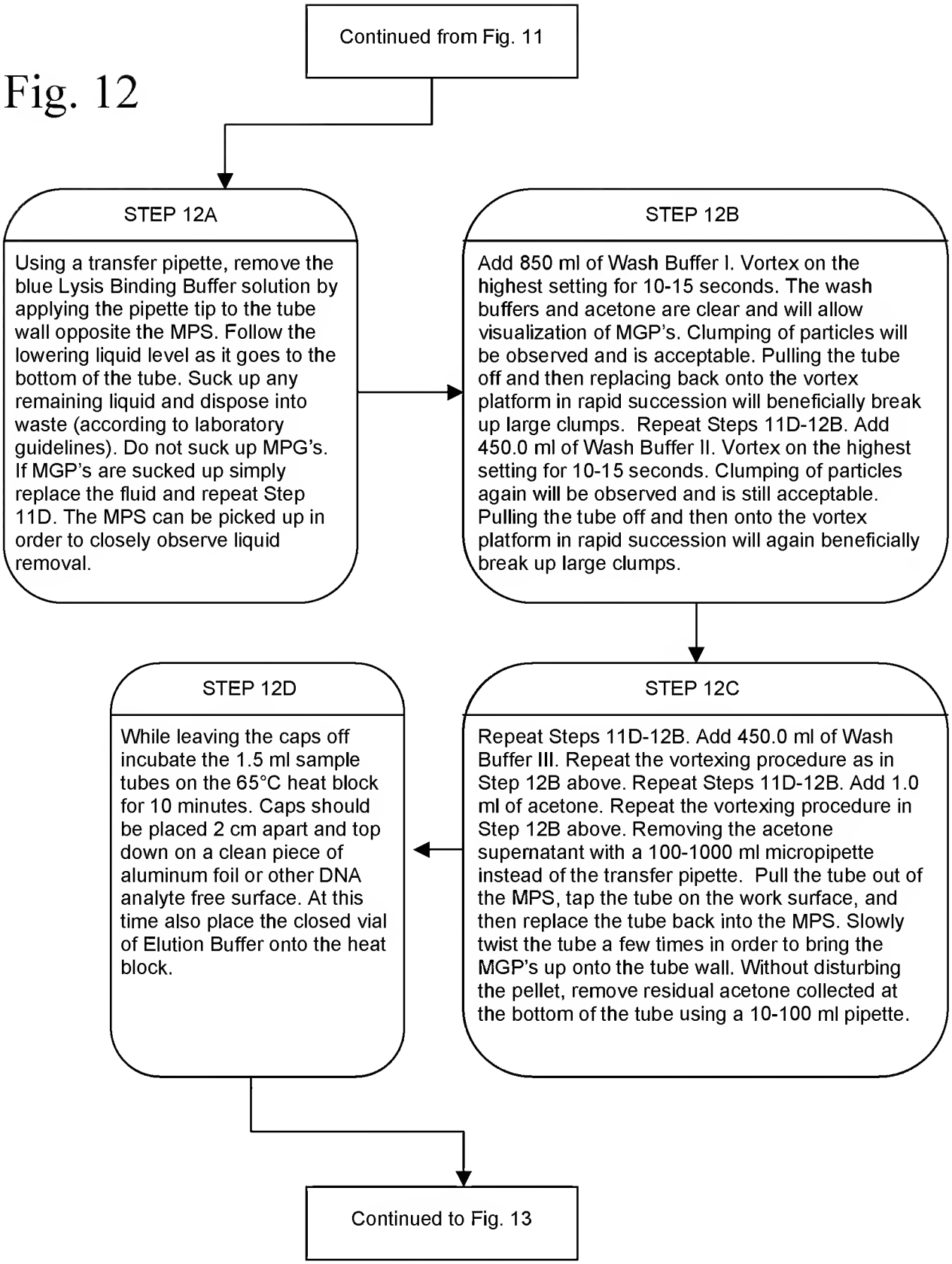
Continued from Fig. 11
STEP 12A
Using a transfer pipette, remove the blue Lysis Binding Buffer solution by applying the pipette tip to the tube wall opposite the MPS. Follow the lowering liquid level as it goes to the bottom of the tube. Suck up any remaining liquid and dispose into waste (according to laboratory guidelines). Do not suck up MPG's. If MGP's are sucked up simply replace the fluid and repeat Step 11D. The MPS can be picked up in order to closely observe liquid removal.
STEP 12B
Add 850 ml of Wash Buffer I. Vortex on the highest setting for 10-15 seconds. The wash buffers and acetone are clear and will allow visualization of MGP's. Clumping of particles will be observed and is acceptable. Pulling the tube off and then replacing back onto the vortex platform in rapid succession will beneficially break up large clumps. Repeat Steps 11D-12B. Add 450.0 ml of Wash Buffer II. Vortex on the highest setting for 10-15 seconds. Clumping of particles again will be observed and is still acceptable. Pulling the tube off and then onto the vortex platform in rapid succession will again beneficially break up large clumps.
STEP 12C
Repeat Steps 11D-12B. Add 450.0 ml of Wash Buffer III. Repeat the vortexing procedure as in Step 12B above. Repeat Steps 11D-12B. Add 1.0 ml of acetone. Repeat the vortexing procedure in Step 12B above. Removing the acetone supernatant with a 100-1000 ml micropipette instead of the transfer pipette. Pull the tube out of the MPS, tap the tube on the work surface, and then replace the tube back into the MPS. Slowly twist the tube a few times in order to bring the MGP's up onto the tube wall. Without disturbing the pellet, remove residual acetone collected at the bottom of the tube using a 10-100 ml pipette.
STEP 12D
While leaving the caps off incubate the 1.5 ml sample tubes on the 65°C heat block for 10 minutes. Caps should be placed 2 cm apart and top down on a clean piece of aluminum foil or other DNA analyte free surface. At this time also place the closed vial of Elution Buffer onto the heat block.
Continued to Fig. 13

Fig. 13

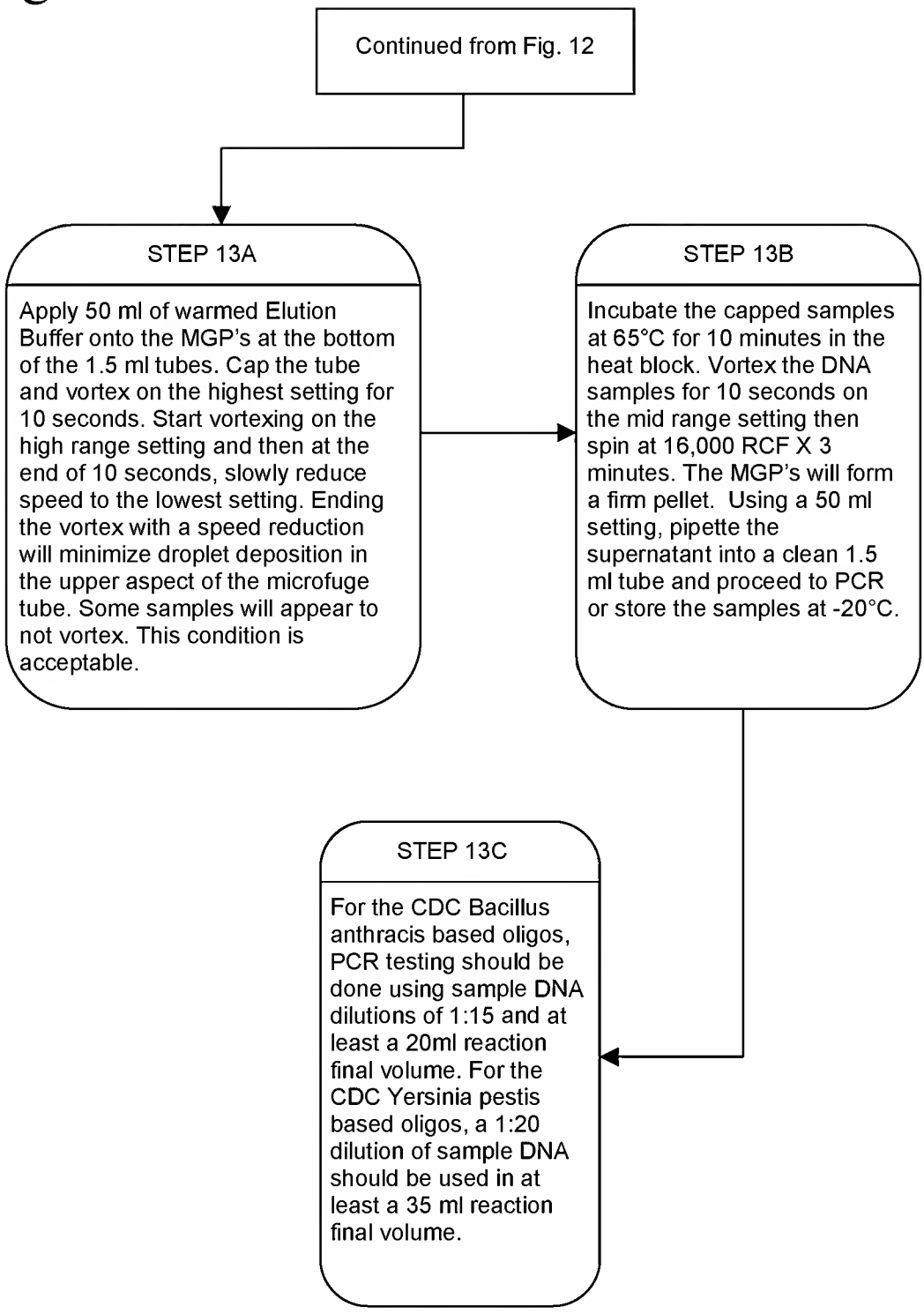
Continued from Fig. 12
STEP 13A
Apply 50 ml of warmed Elution Buffer onto the MGP's at the bottom of the 1.5 ml tubes. Cap the tube and vortex on the highest setting for 10 seconds. Start vortexing on the high range setting and then at the end of 10 seconds, slowly reduce speed to the lowest setting. Ending the vortex with a speed reduction will minimize droplet deposition in the upper aspect of the microfuge tube. Some samples will appear to not vortex. This condition is acceptable.
STEP 13B
Incubate the capped samples at 65°C for 10 minutes in the heat block. Vortex the DNA samples for 10 seconds on the mid range setting then spin at 16,000 RCF X 3 minutes. The MGP's will form a firm pellet. Using a 50 ml setting, pipette the supernatant into a clean 1.5 ml tube and proceed to PCR or store the samples at -20°C.
STEP 13C
For the CDC Bacillus anthracis based oligos, PCR testing should be done using sample DNA dilutions of 1:15 and at least a 20ml reaction final volume. For the CDC Yersinia pestis based oligos, a 1:20 dilution of sample DNA should be used in at least a 35 ml reaction final volume.

Fig. 14a

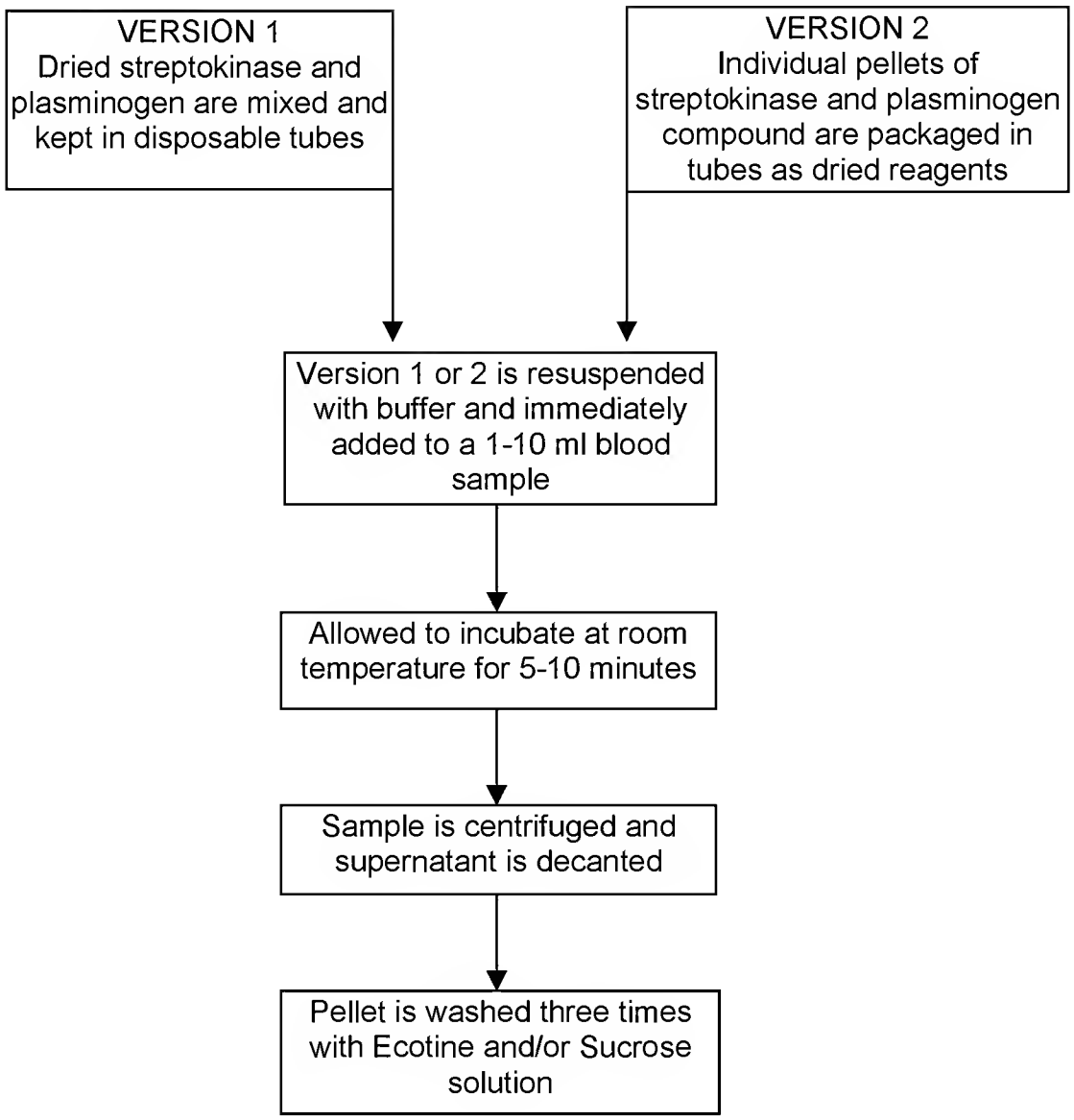
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Sample is centrifuged and supernatant is decanted
Pellet is washed three times with Ecotine and/or Sucrose solution

Fig. 14b

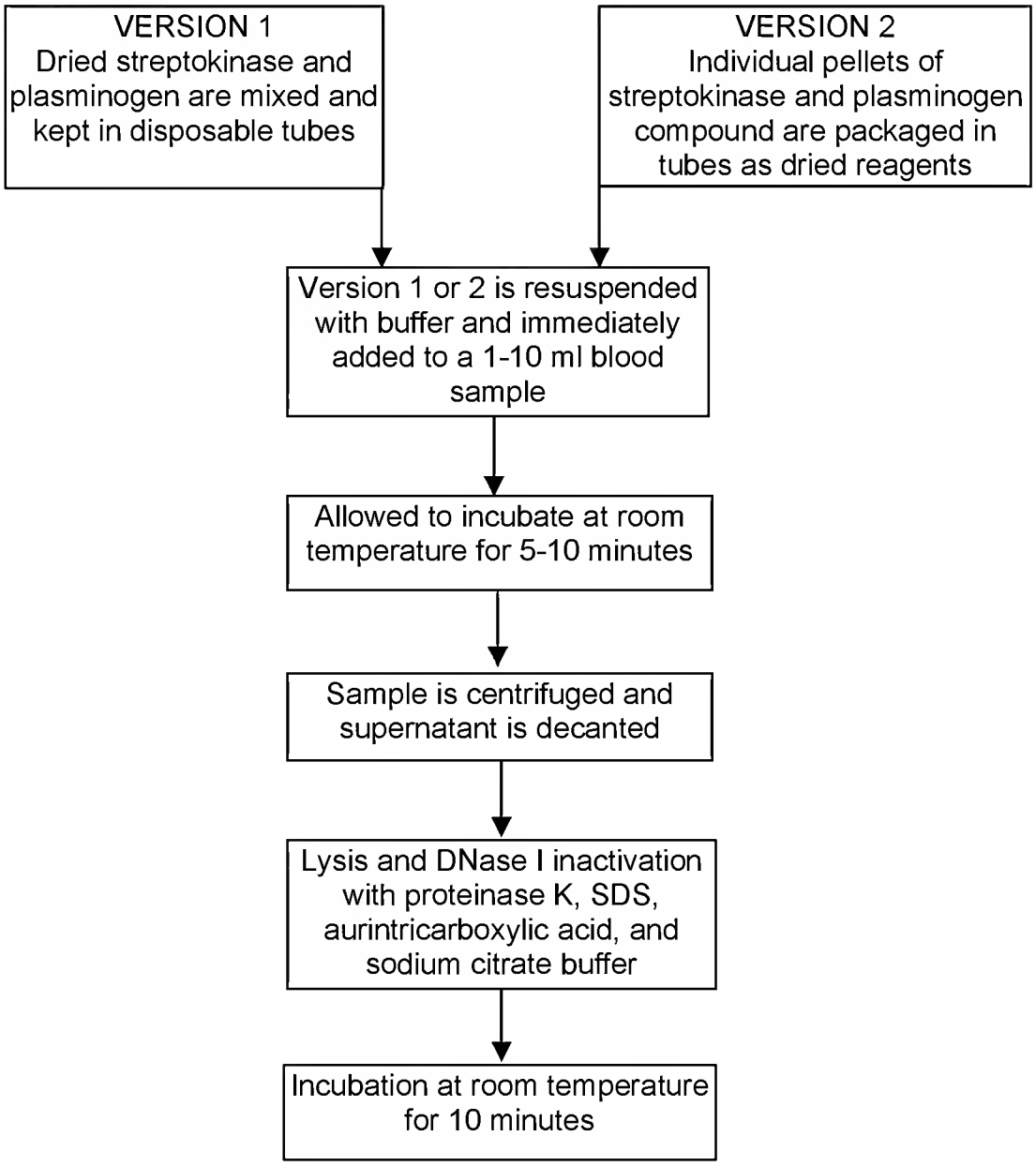
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Sample is centrifuged and supernatant is decanted
Lysis and DNase I inactivation with proteinase K, SDS, aurintricarboxylic acid, and sodium citrate buffer
Incubation at room temperature for 10 minutes

Fig. 15

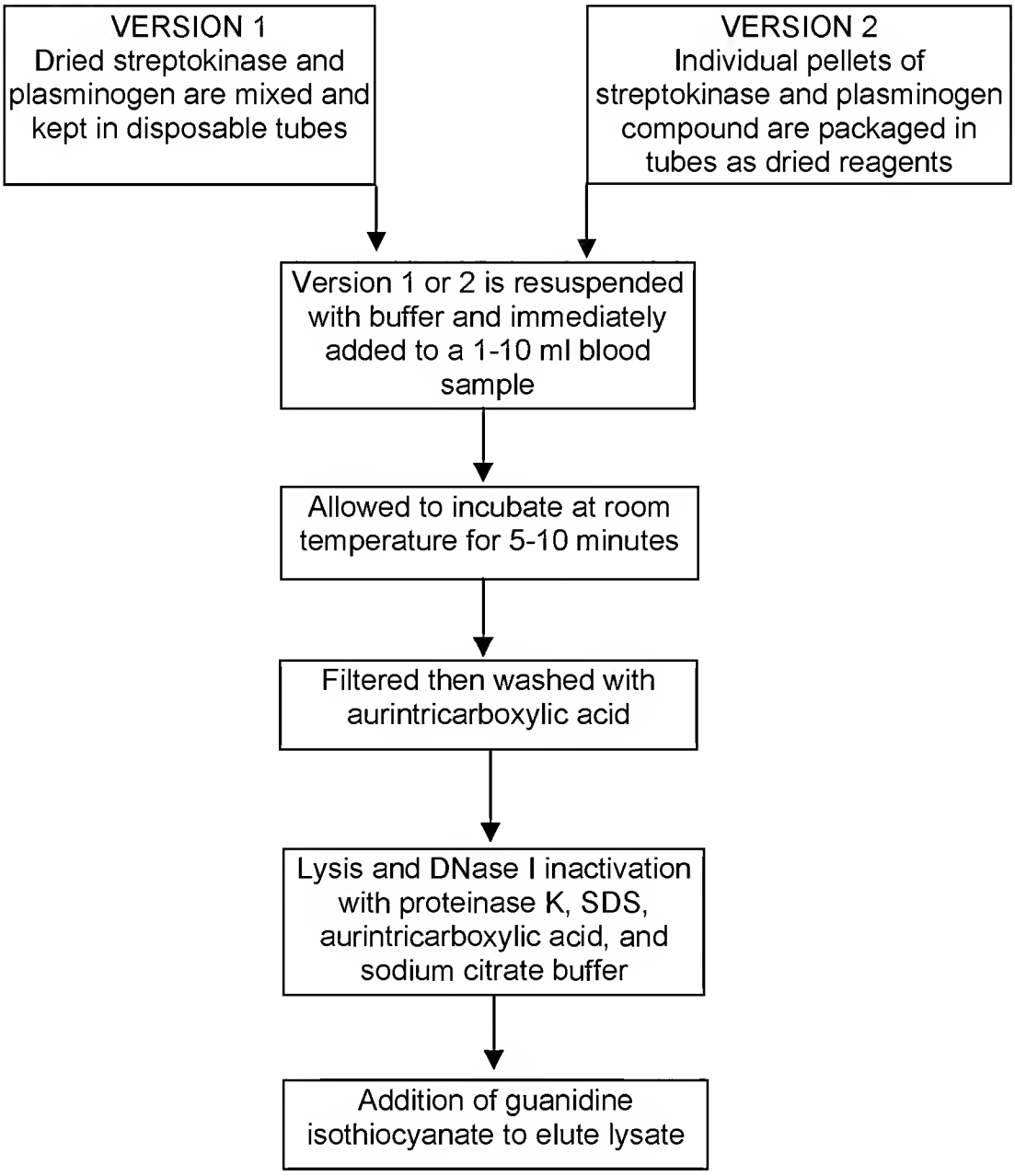
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Filtered then washed with aurintricarboxylic acid
Lysis and DNase I inactivation with proteinase K, SDS, aurintricarboxylic acid, and sodium citrate buffer
Addition of guanidine isothiocyanate to elute lysate

Fig. 16a

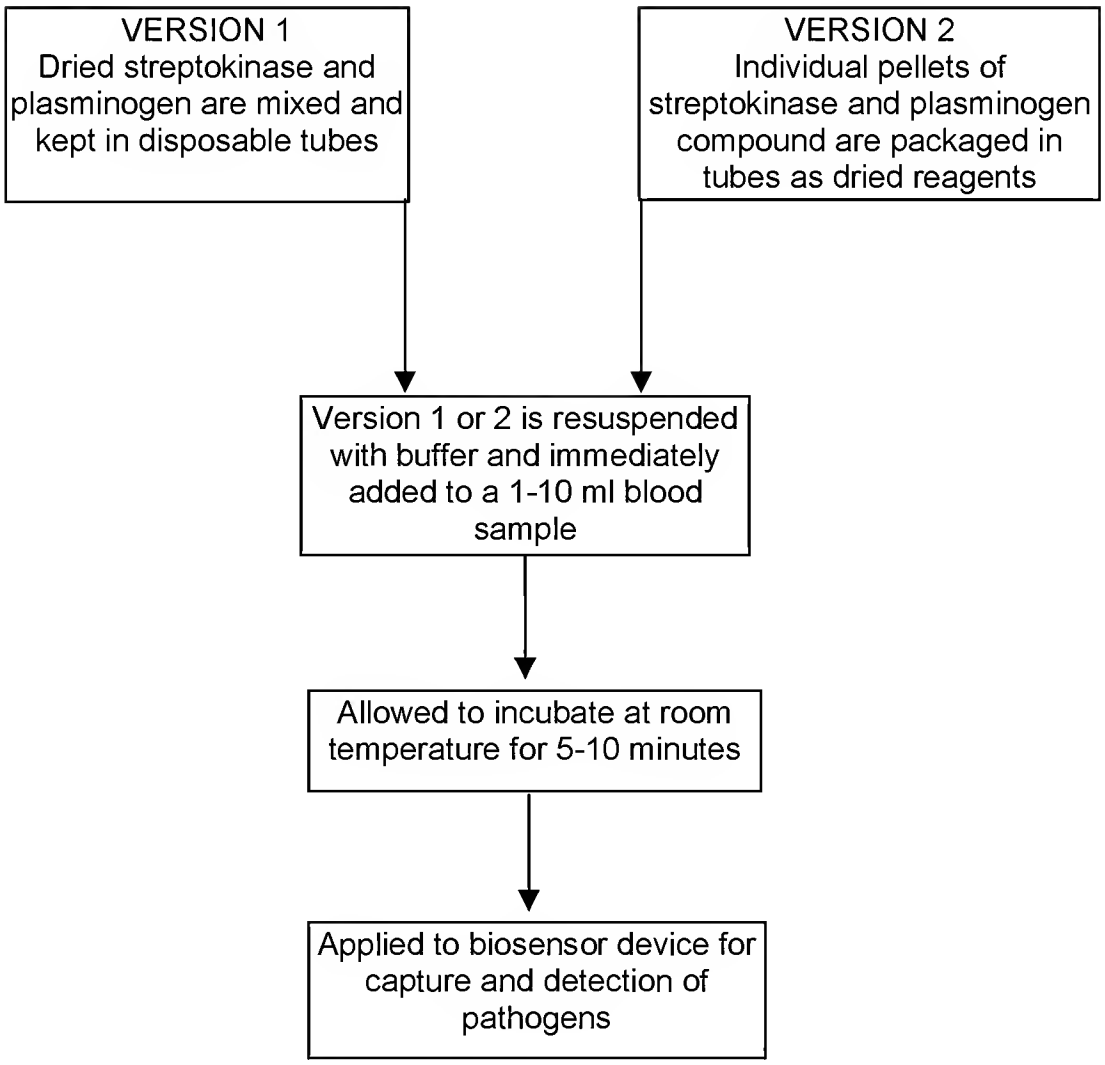
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 is resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Applied to biosensor device for capture and detection of pathogens

Fig. 16b

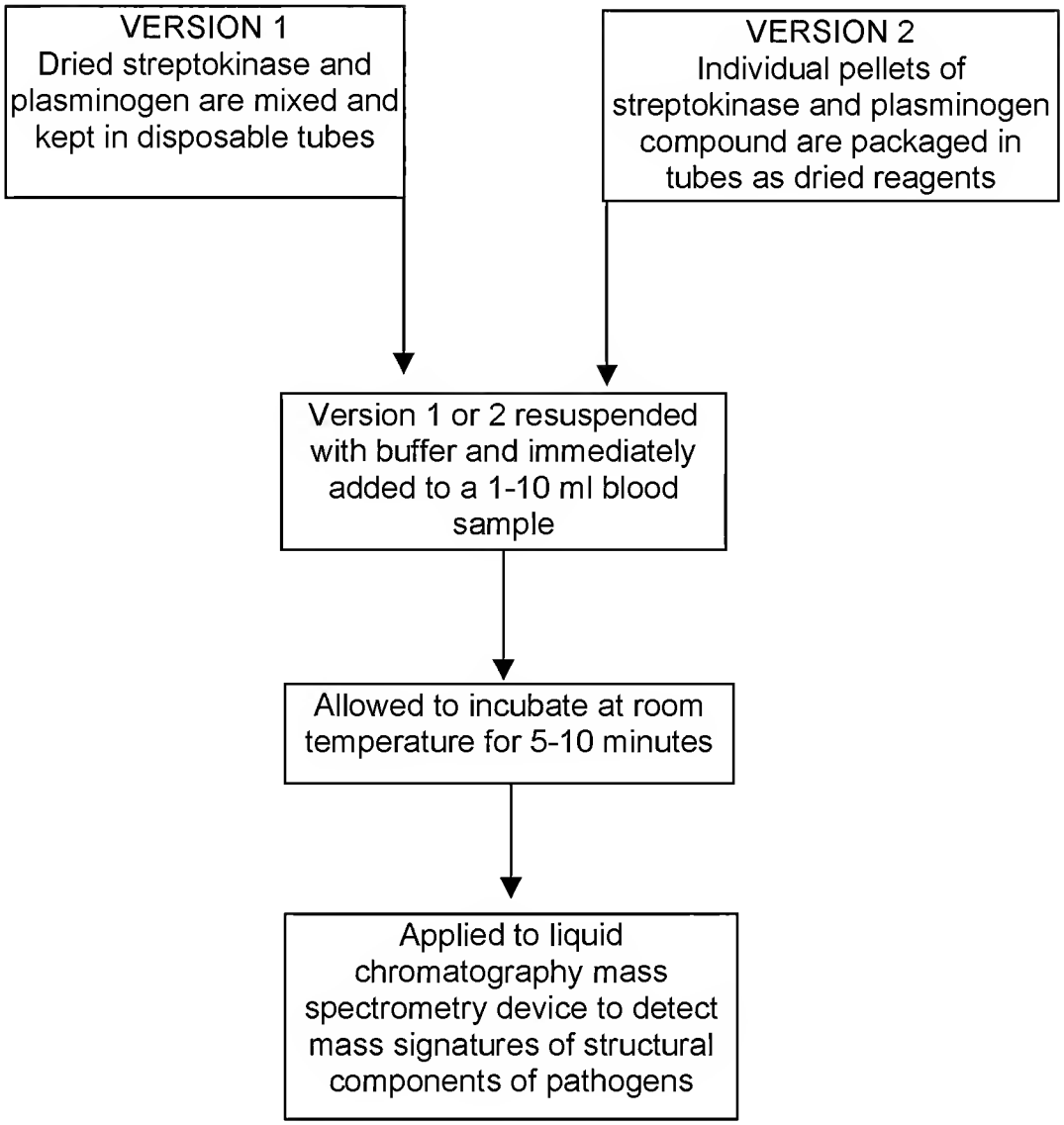
VERSION 1
Dried streptokinase and plasminogen are mixed and kept in disposable tubes
VERSION 2
Individual pellets of streptokinase and plasminogen compound are packaged in tubes as dried reagents
Version 1 or 2 resuspended with buffer and immediately added to a 1-10 ml blood sample
Allowed to incubate at room temperature for 5-10 minutes
Applied to liquid chromatography mass spectrometry device to detect mass signatures of structural components of pathogens

Fig. 17

Noise band crossing points for blood samples spiked with *B. anthracis* and processed with plasminogen, streptokinase, phospholipase A_2, DNase I, and lipase with centrifugation or filtration

	Centrifugation					**Filtration**				
Amount *B. anthracis* Seeded (cfu)	Noise Band Crossing Points			Mean	Std. Dev.	Noise Band Crossing Points			Mean	Std. Dev.
≤ 0.01										
≤ 0.01										
≤ 1.0						40.33	39.89		40.11	
≤ 1.0							37.79		37.79	
≤ 2.0						40.36		37.69	39.03	
≤ 2.0	41.93		40.31	41.12						
≤ 5.0			40.47	40.47		37.90	37.70	37.79	37.80	0.10
≤ 5.0	38.11		40.36	39.24		36.45	36.09	36.81	36.45	0.36
≤ 50.0	37.53	36.24	37.90	37.22	0.87	35.75	34.12	34.98	34.95	0.82
≤ 50.0	36.45	38.15	38.49	37.70	1.09	35.24	34.18	34.68	34.70	0.53

Fig. 18

Sedimentation and solublization of tissue aggregates from 6 ml blood samples exposed to various detergent and enzyme treatments

	Enzyme treatments in a PBS/Triton X-100 buffer						
	Triton X-100 in PBS	Pl.[c] 1U	Ph.[b]	Pl.[c] 1U Ph.[b]	Dn.[a] 1mg	Dn.[a] 1 mg Ph.[b]	Dn.[a] 1 mg Pl.[c] 1U Ph.[b]
% Observable pelleted tissue aggregate post centrifugation	100	100	100	100	90	10	10
Time (min) to solubilization of visible tissue aggregate in BLB[d]	> 360	> 60	> 60	> 60	< 10	< 0.5	< 0.5

[a] DNase I from the Roche MagNa Pure LC DNA Kit III
[b] Phospholipase A_2
[c] Plasminogen and 10K U streptokinase
[d] Bacterial Lysis Buffer from the Roche MagNa Pure LC DNA Kit III

Fig. 19

Filtration characteristics of 6 ml blood samples exposed to various detergent and enzyme treatments

	Enzyme treatments in a PBS/Triton X-100 buffer						
	Triton X-100 in PBS	Dn.[a] 1mg	Dn.[a] 1 mg Ph.[b]	Pl.[c] 5U	Pl.[c] 5U Dn.[a] 1mg Ph.[b]	Pl.[c] 5U Dn.[a] 0.2mg Ph.[b]	Pl.[c] 10U Dn.[a] 0.2mg Ph.[b]
Not filterable	+	+	+				
Filterable with observable tissue aggregates				+		+	
Filterable with out observable aggregates					+		+

[a] DNase I from the Roche MagNa Pure LC DNA Kit III
[b] Phospholipase A_2
[c] Plasminogen converted to plasmin with 10K U streptokinase